UNIVERSIDADE ESTADUAL DE CAMPINAS

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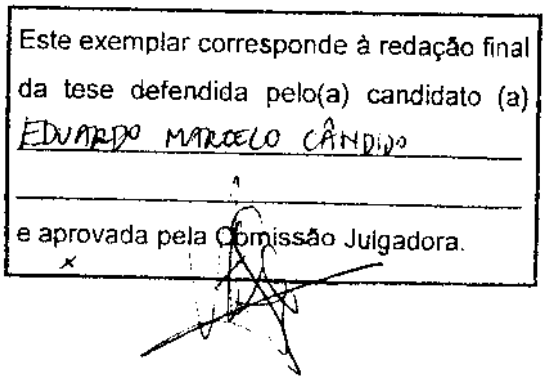
EFEITOS DO ALCOOLISMO CRÔNICO EXPERIMENTAL SOBRE A INTERAÇÃO ESTROMA-EPITÉLIO NO LOBO VENTRAL DA PRÓSTATA DE RATOS UChB



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Orientadora: Profa. Dra. Valéria Helena Alves Cagnon Quitete

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Dedido:

À minha querida família,

meu alicerce e razão do meu viver.

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"Ainda se vier, noites traiçoeiras,

se a cruz pesada for, Cristo estará contigo,

o mundo pode até fazer você chorar,

mas Deus te quer sorrindo!"

(Autor Desconhecido)

SUMÁRIO

RESUMO

O uso prolongado de álcool provoca alterações nocivas ao sistema reprodutor masculino. Atualmente, sabe-se que o estroma é fundamental para a manutenção da homeostase epitélio-estromal da glândula prostática, estando associado a lesões nesse órgão. Assim, o presente trabalho teve como objetivo analisar as alterações morfofuncionais do epitélio secretor e do estroma prostático ocorridas no lobo ventral, bem como a interação epitélio-estromal, associadas à ingestão crônica e à abstinência de álcool. Um total de 30 ratos (10 Wistar e 20 UChB) foram divididos em três grupos experimentais: controle que recebeu água; alcoolista que recebeu etanol diluído a 10º G.L.; e o abstinente que recebeu etanol diluído a 10º G.L. por 120 dias. Após esse período, o grupo abstinente voltou a receber água pura sem etanol. Os três grupos experimentais foram sacrificados após 150 dias de tratamento. Posteriormente, retirou-se a próstata ventral, a qual foi submetida às técnicas de microscopias de luz, eletrônica de transmissão e varredura, imunohistoquímica e dosagem de testosterona sérica associados a parâmetros estereológicos. Os resultados mostraram que os animais alcoolistas apresentaram redução do volume celular epitelial, ocorrência de neoplasia intraepitelial prostática, evaginações epiteliais, hipertrofia estromal e presença de células inflamatórias no estroma prostático. Apesar dos sinais de recuperação epitelial nos animais abstinentes, mantiveram-se as alterações estromais. Assim, pode-se concluir que o etanol é um agente indutor de alterações tanto no epitélio secretor, como no estroma prostático, caracterizando desequilíbrio do microambiente estromal desse órgão, o que certamente o predispõe a processos patológicos.

ABSTRACT

Long-term alcohol treatment has negative effects on prostatic stromal-epithelial interaction. Thus, the aim of the present study was to analyze the histochemical, immunohistochemical and ultrastructural alterations that occur in the prostatic stroma and epithelium of rats submitted to chronic alcohol ingestion and alcohol abstinence, as well as to establish the relationship between these changes and prostatic diseases. Thirty male rats (10 Wistar and 20 UChB rats) were divided into three experimental groups: the control group received tap water, the alcoholic group received ethanol diluted to 10º G.L. for 150 days, and the abstinent group received the same liquid diet as the alcoholic group up to 120 days of treatment and only tap water for 30 days thereafter. At the end of treatment, all animals were sacrificed and the ventral lobe of the prostate was removed and processed for histochemical, immunohistochemical and ultrastructural analyses. In addition, plasma testosterone levels were measured. The results showed prostatic intraepithelial neoplasia, infolding of the epithelium towards the stroma, stromal hypertrophy and the presence of inflammatory cells in alcoholic animals. In the abstinent group, alterations were noted mainly in the stromal area. In conclusion, ethanol triggers alterations in prostatic epithelial and stromal compartments, affecting the stromal microenvironment and predisposing the organ to pathological processes.

O alcoolismo é uma doença de extensão mundial, sendo um dos freqüentes diagnósticos encontrados em casos clínicos levando os indivíduos à morbidade e morte prematuras (Caces et al.,1995; Campbell et al., 1996, Foroud & Li, 1999). Nos Estados Unidos, o consumo excessivo de álcool é a terceira principal causa evitável de morte e está associado a conseqüências adversas à saúde, como cirrose hepática e diferentes tipos de cânceres (Centers for Disease Control and Prevention, 2004).

O álcool (etanol, metanol e n-propanol) é absorvido rapidamente no estômago, intestinos delgado e grosso, nos pulmões (vapor de álcool), na bexiga urinária, cavidades peritonial e pleural. Sua distribuição ocorre através de difusão rápida e uniforme por capilares e membranas para atingir todos os tecidos e líquidos extracelulares do organismo (Kalant, 1983). A excreção corpórea do álcool é dada pela soma das taxas de eliminação através da urina, da respiração e do suor, o que representa cinco por cento do total a ser eliminado. A principal via excretora ocorre no fígado através das ações de enzimas oxidativas: álcool dehidrogenase, a catalase e o sistema de oxidações metabólicas microssomais (Kalant, 1983).

O álcool e seus metabólitos provocam distúrbios generalizados, especialmente, nos sistemas nervoso central, reprodutor masculino e órgãos hematopoiéticos (Marks & Wright, 1978; Martinez et al., 2001). Atua também, diretamente no fígado, pâncreas, intestinos, rins, glândulas endócrinas e glândulas sexuais acessórias (Marks & Wright, 1978; Martinez et al., 2001; Gomes et al., 2002). É importante informar que, todos os caminhos do metabolismo de etanol, resultam na produção de acetaldeído (Lieber, 1987). O acetaldeído, por sua vez, é uma substância altamente tóxica, mutagênica e carcinogênica (Pöschl &

Seitz, 2004). Ele interfere em muitos sítios onde há síntese e reparo de DNA podendo, conseqüentemente, resultar no desenvolvimento de um tumor (Pöschl & Seitz, 2004). De acordo com a Agência Internacional de Pesquisa sobre o Câncer (International Agency for Research on Cancer – IARC), há evidências suficientes para identificar o acetaldeído como um carcinógeno, ao menos, em animais experimentais (Anonymous, 1985).

O uso de modelo animal tem elucidado muitos aspectos bioquímicos, fisiológicos e morfológicos envolvidos no hábito da ingestão de bebidas alcoólicas por seres humanos. Desde 1926, caracterizou-se que roedores escolhem voluntariamente etanol diluído em diferentes concentrações à água, sendo esta preferência influenciada por fatores ambientais, fisiológicos e comportamentais (Richter, 1926; Cícero & Badger, 1977). Além desses fatores, atualmente é conhecido que o alcoolismo está ligado a desordem genética complexa, resultado da ação múltipla da interação entre genes (Foroud & Li, 1999). Diversos estudos, em camundongos (Erwin et al., 1980) e ratos (Tabakoff & Ritzmann, 1979; Waller et al., 1983) demonstraram a preferência alcoólica desses e a diminuição da sensibilidade inicial aos efeitos do álcool. Assim como, é o caso da linhagem de ratos UChB, os quais apresentam aguda e rápida tolerância ao etanol (Tampier & Mardones, 1999). Esses ratos, originados a partir de ratos Wistar, passaram por décadas de seleção através de cruzamentos consangüíneos entre animais com baixo e alto consumo voluntário de etanol à 10%, recebendo a denominação UChA e UChB, respectivamente (Martinez et al., 2001).

No sistema reprodutor uma das conseqüências do alcoolismo é o hipogonadismo, que ocorre tanto no homem como em animais de laboratório (Bannister & Lowosky, 1987). Alguns autores acreditavam que essa disfunção sexual estaria relacionada à doença crônica do fígado (Brown et al., 1964; Van Thiel et al., 1974; Van Thiel & Lester, 1979), já que,

alterações relativas ao sistema reprodutor estavam exacerbadas em indivíduos com cirrose alcoólica (Van Thiel et al., 1975; Wang et al., 1991). No entanto, um estudo recente realizado por Tadic e et al. (2000), demonstrou o oposto, apresentando dados claros e convincentes de que o hipogonadismo precede a hiperestrogenização e injúria hepática, que seriam provocadas pela profunda redução dos níveis de testosterona sérica, resultando na perda de funções hepáticas reguladas por andrógenos, tais como, a atividade das enzimas de metabolização do estrógeno e a atividade dos receptores de andrógenos. A diminuição da atividade das enzimas hepáticas de metabolização do estrógeno resulta na elevação dos níveis séricos de estradiol e conseqüente alteração da taxa estradiol/testosterona em alcoólatras crônicos. Isto posto, acredita-se que o etanol seja uma toxina que atua de forma direta sobre as gônadas, alterando a síntese de testosterona testicular (Gary et al., 1976; Elligboe & Carole, 1979; Rivier & Vale, 1983; Bannister & Lowosky, 1987; Anderson et al., 1989; Saxena et al., 1990; Tadic, et al., 2000) e também indiretamente, provocando efeitos deletérios sobre o eixo hipotálamo-hipófise-gônada (Van Thiel, 1983) e no metabolismo do fígado (Palmer, 1989; Tadic, et al., 2000). Na próstata ventral de roedores, acredita-se tanto no efeito direto como no indireto do etanol sobre o epitélio glandular de roedores (Martinez et. al., 1993).

A influência nociva do álcool sobre o sistema reprodutor masculino foi demonstrada com variadas metodologias para a indução do alcoolismo. As principais alterações morfológicas são: lesões das células germinativas testiculares, diminuição dos diâmetros dos túbulos seminíferos, depressão no nível de testosterona sérica, redução do peso das glândulas sexuais acessórias, diminuição significativa nas alturas das células dos epitélios secretores das vesículas seminais, próstatas ventral, lateral e dorsal (Van Thiel et al., 1979; Semczuk & Rzeszowska 1981; Willis et al., 1983; Anderson et al., 1985; Salonen &

Huhtaniemi 1990; Martinez et al., 1993; Cagnon et al., 1996; Martinez et al.,1997; Cagnon et al., 1998; Garcia et al.,1999; Cagnon et al., 2001). Nas glândulas sexuais acessórias, destacam-se os efeitos deletérios sobre as organelas envolvidas no processo secretor como dilatação das cisternas do retículo endoplasmático granular e do complexo de Golgi, ruptura dos microvilos e acúmulo de gotas lipídicas no citoplasma das células epiteliais (Martinez et al. 1993; Cagnon et al., 1996; Martinez et al.,1997; Cagnon et al., 1998; Garcia et al., 1999; Cagnon et al., 2001).

A próstata é a glândula sexual acessória masculina que desperta interesse entre os pesquisadores, pois freqüentemente é acometida por diferentes doenças como hiperplasia prostática benigna e câncer (Guess, 2001). Estudos epidemiológicos têm demonstrado que os casos de cânceres prostáticos vêm aumentando desde 1998, sendo que muitos levam o paciente a óbito (Landis et al., 1998). Nos roedores, observou-se incidência do mesmo no lobo ventral da próstata de ratos e, ocasionalmente, no lobo dorsal. É conhecido que as doenças prostáticas, especialmente o carcinoma, podem ter natureza endócrina (Morton et al., 1996) e que a ocorrência aumenta com a idade (Davies & Eaton, 1991).

As secreções da próstata e da vesícula seminal têm fundamental importância no processo reprodutivo (Price & Williams-Ashman, 1961; Cavazos, 1975; Mann & Lutwak-Mann, 1981). Elas são compostas por diversos nutrientes que compõem o líquido seminal, fluido essencial para a nutrição e motilidade dos espermatozóides. A próstata secreta proteínas, entre as quais há grande variedade de enzimas, carboidratos e outras substâncias, incluindo o citrato. Dentre as enzimas, acreditava-se que a fosfatase ácida fosse exclusivamente secretada pela próstata. Contudo, tem sido detectada nos diferentes tipos de tecidos como fígado, testículos, baço e rins, além da próstata (Tenniswood et al., 1975). É sabido que, esta enzima age em uma variedade de substratos como fenilfosfato, α-naftil

fosfato, paranitro fenil fosfato, participando portanto do mecanismo básico para a hidrólise desses ésteres de fosfato (Blandy & Lytton, 1986). A síntese da fosfatase ácida é andrógeno dependente (Tenniswood et al., 1975). A atividade funcional desta foi detectada em várias espécies incluindo o homem (Costello & Franklin, 1994).

Diferentes estudos, realizados em roedores, demonstram que a morfogênese, manutenção da atividade funcional e morfologia (Lung & Cunha, 1981), proliferação e diferenciação (Rajfer & Coffey, 1978; Okamoto et al., 1982; Davis & Eaton, 1991) das células das glândulas sexuais acessórias são reguladas por andrógenos testiculares. A testosterona é o principal andrógeno a induzir a diferenciação da vesícula seminal (Tsuji et al., 1994) e da próstata (Costello & Franklin, 1994). Além da testosterona, acredita-se que outros hormônios estejam implicados no processo de crescimento e funcionamento da próstata, como a prolactina, que atua sinergicamente à testosterona e o estrógeno, cujo mecanismo de atuação não está totalmente esclarecido (Cunha et al., 1987; Cunha et al., 2003; Risbridger et al., 2003). Contudo, estudos revelam que o desenvolvimento da próstata é regulado principalmente pela dihidrotestosterona (Tsuji et al., 1994; Ekman, 2000), o principal andrógeno tecidual (Hsing, 2001). A próstata é o maior sítio de produção não testicular de dihidrotestosterona, onde a testosterona é convertida irreversivelmente à dihidrotestosterona através da ação da enzima 5 α-redutase Tipo 2 (Hsing, 2001). A castração é um dos métodos mais utilizados para o entendimento dos mecanismos envolvendo a testosterona na manutenção e no funcionamento das glândulas sexuais acessórias. É conhecido que, essa condição induz a atrofia dos epitélios secretores da vesícula seminal, próstata e glândula de coagulação (Wakade et al., 1975, Sjöstrand &

Swendin, 1976), bem como leva a intensa reorganização da matriz extracelular envolvendo as fibras colágenas e ativação das células musculares lisas (Vilamaior et al., 2000).

Nos roedores, a próstata é uma glândula complexa formada por três pares de lobos: ventral, lateral e dorsal (Langworthy, 1965; Purinton et al., 1972; Vaalasti & Hervonen, 1979; Jesik et al., 1982; Prins, 1992), os quais drenam suas secreções, através de ductos, para a uretra pélvica (Jesik et al., 1982; Cavazos, 1975). Quanto à sensibilidade androgênica, considerável heterogeneidade é observada entre os diferentes lobos prostáticos (Prins, 1992).

A próstata ventral é constituída de ácinos revestidos por epitélio simples. Cada ácino consiste de células colunares assentadas em nítida membrana basal (Cavazos, 1975). Os componentes predominantes da membrana basal são a laminina, o colágeno do tipo IV e o proteoglicano heparan sulfato (Jorcyk et al., 1998). O estroma prostático é um arranjo complexo de células estromais e matriz extracelular associadas a fatores de crescimento, moléculas reguladoras e enzimas de remodelação. Vasos sanguíneos, terminações nervosas e células imunes também constituem partes integrais do estroma (Tuxhorn, et al., 2001). Fibroblastos e células musculares lisas são os principais tipos celulares na próstata humana. A principal função destas células é sintetizar componentes estruturais e reguladores da matriz extracelular. Esta, por sua vez, é uma rede de proteínas fibrilares, glicoproteínas adesivas e proteoglicanas (Kreis & Vale, 1999; Tuxhorn, et al., 2001). Além disso, é um reservatório de fatores de crescimento ativos e latentes (Taipale & Keski-Oja, 1997; Tuxhorn, et al., 2001). Componentes estruturais, tais como o colágeno e fibras elásticas, proporcionam rigidez mecânica e flexibilidade ao tecido, servindo também como substrato para adesão celular e migração, as quais são mediadas por glicoproteínas adesivas, tais como laminina e fibronectina. Proteoglicanas regulam a estrutura e permeabilidade da

matriz extracelular, se ligando a fatores de crescimento, proteases e inibidores de proteases, modulando a atividade destes (Taipale & Keski-Oja, 1997; Kreis & Vale, 1999 e Tuxhorn, et al., 2001). Juntos células estromais e matriz extracelular criam um microambiente que regula o crescimento e diferenciação funcional das células adjacentes, desempenhando cada um desses elementos, importante papel na manutenção da forma e função tecidual (Narbaitz, 1975; Labat-Robert et al., 1990; Tuxhorn, et al., 2001). É importante destacar que, na maioria dos tecidos o colágeno do tipo I é o mais abundante, representando o principal componente fibrilar (Bateman et al., 1996). O colágeno tipo III, também é um dos elementos fibrilares e geralmente está co-localizado com o do tipo I (Liu et al., 1997), sendo o principal constituinte das fibras reticulares (Vilamaior et al., 2000). A interação estroma-epitélio tem importante papel na manutenção da estrutura e funcionamento da glândula prostática (Ekman,2000) e pode ser considerada, baseando-se em aspectos morfológico, funcional e embriológico, como formando uma única unidade funcional (Aumuller & Seitz, 1990). Estudos recentes tem demonstrado a interação entre estroma e epitélio como fator primordial na progressão do carcinoma prostático (Tuxhorn, et al., 2001, Cunha & Matrisian, 2002). O desenvolvimento de um microambiente estromal alterado em resposta ao carcinoma é uma característica comum de muitos tumores (Tuxhorn, et al., 2001). Em adição, têm sido enfatizado o papel das metaloproteinases da matriz extracelular, estando estas envolvidas em uma ampla variedade de funções que podem assistir a iniciação do tumor, crescimento, migração, angiogênese, seleção de subpopulações resistentes a apoptose, invasão e metástase. Desta forma, esta família enzimática atualmente, tem sido vista não somente como “destruidoras” dos elementos da matriz extracelular, mas como parte de um refinado sistema de comunicação através do qual o tumor interage com o estroma (Lynch, 2002). Assim, estudos adicionais sobre os

mecanismos celulares e moleculares da interação entre células estromais e cancerosas podem promover novas estratégias terapêuticas para regulação do crescimento tumoral, beneficiando pacientes que sofrem de câncer (Cunha, 2002).

Ultra-estruturalmente, as células secretoras do lobo ventral da próstata apresentam proeminente retículo endoplasmático granular ocupando grande área do citoplasma, com cisternas paralelas e achatadas. Além disso, o complexo de Golgi é bem desenvolvido e grânulos de secreção são caracterizados nas regiões supranuclear e apical do citoplasma, respectivamente. Pequenos microvilos revestem a superfície da célula voltada para o lúmen (Dahl et al., 1973; Cavazos, 1975).

JUSTIFICATIVA E OBJETIVOS

A toxicidade do álcool sobre a próstata leva a complexas conseqüências nesse órgão e dúvidas persistem a respeito da morfofisiologia estromal e sua interação com células epiteliais frente ao alcoolismo crônico. Assim sendo, o objetivo do presente estudo foi analisar as alterações morfofuncionais na porção secretora e estromal do lobo ventral da próstata de ratos UChB frente ao uso crônico de álcool e à abstinência alcoólica, além de associar esses resultados ao surgimento de lesões prostáticas.

5.1 – Procedimento experimental:

Um total de 20 ratos machos (*UChB*) e 10 ratos machos (*Wistar*) com três meses de idade foram utilizados; provenientes do Departamento de Anatomia/IBB/UNESP e Centro de Bioterismo da Unicamp – CEMIB, respectivamente; mantidos no Biotério do Departamento de Anatomia/IB/UNICAMP.

Os animais foram divididos em três grupos experimentais: controle, alcoolista, e abstinente. O grupo **controle** (10 animais-*Wistar*) recebeu somente água. O grupo **alcoolista** (10 animais-*UChB*) recebeu etanol diluído a 10º *Gay Lussac* (10% v/v). O grupo **abstinente** (10 animais-*UChB*) recebeu etanol diluído à 10º *Gay Lussac* ao longo de 120 dias. Após 120 dias do início da ingestão de etanol, realizou-se a retirada gradativa do etanol, mantendo esse tratamento ao longo de 30 dias, totalizando 150 dias de tratamento, tendo recebido, portanto, dieta similar ao grupo controle durante o período de abstinência. Os grupos alcoolista e controle foram sacrificados após 150 dias de tratamento. Os três grupos experimentais receberam dieta em grãos balanceada (Nuvilab®) e água "*ad libitum*". É importante informar que, 1g da dieta balanceada fornece 2,7 kcal de energia e 1g de etanol possui 7,1 Kcal. Todos os animais foram mantidos individualmente em gaiolas plásticas. Diariamente, realizou-se mensurações das ingestões de água e/ ou água + etanol, e mensurações semanais do consumo da dieta em grãos balanceada, além do peso corpóreo dos animais.

Após 150 dias de tratamento, todos os animais foram pesados em balança semi-analítica Marte AS 5500 e sacrificados. Amostras do lobo ventral foram coletadas para

análises macroscópica, histoquímica, imunohistoquímica e de microscopia eletrônica de transmissão e varredura.

Nos grupos alcoolistas, antecedendo o início do experimento, foram administradas doses crescentes de etanol na escala de diluição: 5% a 10%. As diluições alcoólicas tiveram duração de 7 dias. A administração gradativa do etanol teve como objetivo a adaptação dos animais ao modelo experimental.

O modelo de alcoolismo utilizado foi o voluntário, no qual forneceu-se etanol diluído em água, além de água pura e dieta sólida em grãos aos animais (Martinez et al., 2000).

5.2 – Macroscopia

Os animais dos grupos experimentais foram anestesiados com Francotar®/Virbaxyl® (Virbac-Brazil) (1:1) na dosagem de 0,25 ml para cada 100 gramas de peso corpóreo. A seguir, foi realizada uma incisão na parede abdomino-pélvica, expondo o aparelho urogenital. Parte desse complexo que inclui próstata, glândulas de coagulação, vesículas seminais, uretra, bexiga urinária, testículos, ureteres e ductos deferentes, foi dissecado com auxílio do microscópio cirúrgico DF Vasconcelos.

5.3 – Microscopia de Luz

As amostras do lobo ventral foram coletadas de cinco animais de cada grupo experimental e fixadas em solução de Bouin e formaldeído a 10%. Após fixação, passaram pela rotina histológica usual até serem incluídas em paraplástico (Paraplast Plus/Oxford) e resina de metacrilato (Hitoresin Embedding Kit, Leica, USA). A secção do material foi realizada em micrótomo (Biomet – modelo 1130) para a obtenção de cortes com 3μm

espessura. Finalmente, o material foi corado nas colorações Hematoxilina-Eosina, Picrossírius-hematoxilina (Junqueira et al., 1979) e Reticulina de Gömori (Vilamaior et al., 2000) e fotografado no fotomicroscópio (Nikon Eclipse E-400), no laboratório do Departamento de Anatomia/IB/Unicamp.

5.4 – Imunohistoquímica para Fibras Colágenas

As amostras do lobo ventral foram coletadas de cinco animais de cada grupo experimental, embebidas em meio de inclusão para baixas e médias temperaturas e congelados em nitrogênio líquido. A seguir foram obtidos cortes com 7 μm de espessura no criostato Mícron HM505E, coletados em lâminas silanizadas e fixados, primeiramente, em metanol gelado (3 min) e acetona gelada (3 min), secados ao ar por 30 min com posterior fixação em paraformaldeído (4%) por 10 min., e lavados com PBS. A seguir, as amostras foram incubadas com solução bloqueadora (4% BSA e 1% Triton X-100 em PBS; Sigma, St. Louis, MO) por 1 hora em temperatura ambiente para bloqueio de ligação inespecíficas. A seguir, o material foi incubado com o anticorpo primário anti colágeno I e III (coelho) (Chemicon International -Inc., Temecula, CA), diluído em solução bloqueadora de acordo com as instruções do fabricante por 12 horas à 4ºC. Posteriormente, as lâminas com as amostras foram lavadas (3X de 5 minutos) em tampão PBS, com posterior incubação no anticorpo secundário anti-Ig de coelho conjugado à fluoresceína (cabra), diluído 1:100 em solução bloqueadora. As lâminas foram montadas em DABCO (Sigma Chemical CO) com solução contendo 5 μg de DAPI (Sigma Chemical CO) com posterior observação no microscópio confocal BioradMRC1024UV. Todas as reações imunohistoquímicas foram realizadas no laboratório do Departamento de Anatomia/IB/Unicamp.

5.5 – Estereologia

Cinco ratos de cada grupo foram utilizados. As áreas epiteliais e estromais no lobo ventral da próstata foram medidas (25 campos por animal). O aumento utilizado na captação das imagens para a análise morfométrica foi o de 200x. As áreas foram medidas utilizando-se o sistema de análise de imagens computadorizadas (Image-Pro Express 4.0). Os volumes celulares, citoplasmáticos e nucleares foram medidos em cortes corados para estudo em microscopia de luz (histoquímica). Os dados para o volume nuclear foram obtidos através da média de 100 mensurações por animal. Foram medidos os diâmetros maior (D) e menor (d) e a média do volume foi calculada considerando os núcleos elipsóides. As medidas foram realizadas com auxílio da ocular graduada (10x) acoplada ao microscópio de luz Zeiss, fixando-se as observações com objetiva de 100x. A calibragem foi realizada através de lâmina graduada com divisões de 0,01 mm (10 μm) para obtenção da unidade em micrômetros. Para a determinação do volume citoplasmático, uma ocular contendo um retículo de integração quadrilátera com 100 pontos acoplada ao microscópio de luz Zeiss e objetiva de 100x foi utilizada. Pontos no citoplasma e núcleo foram contados em 10 áreas por grupo e as frações citoplasmática e nuclear foram obtidas (Weibel, 1979). Estes dados e o volume nuclear foram utilizados para estimar o volume citoplasmático de cada animal. O volume celular foi calculado através da somatória dos volumes nuclear e citoplasmático.

5.6 – Microscopia Eletrônica de Transmissão

Um total de cinco animais, de cada grupo experimental, foram perfundidos com glutaraldeído a 2,5%, através do ventrículo esquerdo do coração (Sprando,1990). As próstatas ventrais foram coletadas e fragmentadas, até que tivessem cerca de 0,5 mm de

espessura. Em seguida, os fragmentos foram submetidos ao complemento de fixação, por imersão no mesmo fixador e pós-fixados em tetróxido de ósmio a 1% por duas horas. Os fragmentos passaram então por uma série crescente de acetona, foram incluídos em resina plástica (Polyscience/USA) e cortados com 0,5 micrômetro de espessura, no ultramicrótomo LKB 8800 ultratome III com navalha de vidro e corados com azul de toluidina (1%). Os cortes corados foram utilizados para definir áreas específicas para o estudo em microscopia eletrônica de transmissão tanto do epitélio secretor quando do estroma prostático. Após a análise, os blocos foram aparados (trimagem) e submetidos a ultramicrotomia com navalha de diamante no ultramicrótomo Ultracult UCT 020 Leica. Os cortes obtidos foram montados em telas de cobre de 200 mesh e contrastados pelo acetato de uranila (Watson, 1958) e pelo citrato de chumbo (Reynolds, 1963), examinados e fotografados no microscópio eletrônico de transmissão LEO 906 no Laboratório de Microscopia Eletrônica do Instituto de Biologia da Unicamp.

5.7 – Microscopia Eletrônica de Varredura

As amostras foram preparadas como para a microscopia eletrônica de transmissão até a desidratação final em acetona absoluta, sendo posteriormente submetidas à secagem em ponto crítico com CO_2 líquido utilizando-se o aparelho CPD 030 Balzers. As amostras foram então cobertas com ouro utilizando-se o aparelho SCD 050 Balzers e observadas no microscópio eletrônico de varredura JEOL JSM-5880LV.

5.8 – Dosagem Hormonal

A análise quantitativa do nível de testosterona total sérica foi avaliada através de radioimunoensaio (Testosterona Total/Kit COAT-A-COUNT Diagnostic Procts Corporation Los Angeles, CA, USA).

5.9 – Análise Estatística

O estudo da comparação entre grupos experimentais foi realizado para as variáveis: variação de peso (ΔP) = peso final – peso inicial (g), consumo diário da dieta em grãos balanceada (g), consumo diário de água (ml), além do cálculo da ingestão de etanol por kg de peso corpóreo por dia, realizados através da técnica de análise de variância. Foram também avaliados os pesos testicular e das glândulas de coagulação e vesículas seminais. A análise estatística foi complementada com o teste de comparações múltiplas de Tukey para comparação entre as médias (Norman & Streiner, 1994). Todas as conclusões no presente estudo foram realizadas no nível de 5% de significância.

ARTIGO

O artigo “Experimental alcoholism and pathogenesis of prostatic diseases in UChB rats” será submetido à revista *The Prostate*.

“EXPERIMENTAL ALCOHOLISM AND PATHOGENESIS OF PROSTATIC DISEASES IN UChB RATS”

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Short title: Alcoholism and prostatic pathogenesis.

Key words: alcoholism, UChB rats, prostate, stromal-epithelial interaction, morphology.

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ABSTRACT

Long-term alcohol treatment has negative effects on prostatic stromal-epithelial interaction. Thus, the aim of the present study was to analyze the histochemical, immunohistochemical and ultrastructural alterations that occur in the prostatic stroma and epithelium of rats submitted to chronic alcohol ingestion and alcohol abstinence, as well as to establish the relationship between these changes and prostatic diseases. Thirty male rats (10 Wistar and 20 UChB rats) were divided into three experimental groups: the control group received tap water, the alcoholic group received ethanol diluted to 10º G.L. for 150 days, and the abstinent group received the same liquid diet as the alcoholic group up to 120 days of treatment and only tap water for 30 days thereafter. At the end of treatment, all animals were sacrificed and the ventral lobe of the prostate was removed and processed for histochemical, immunohistochemical and ultrastructural analyses. In addition, plasma testosterone levels were measured. The results showed prostatic intraepithelial neoplasia, infolding of the epithelium towards the stroma, stromal hypertrophy and the presence of inflammatory cells in alcoholic animals. In the abstinent group, alterations were noted mainly in the stromal area. In conclusion, ethanol triggers alterations in prostatic epithelial and stromal compartments, affecting the stromal microenvironment and predisposing the organ to pathological processes.

INTRODUCTION

Alcoholism is a disease found worldwide and is one of the most frequent clinical diagnoses, causing morbidity and premature death [1-3]. In the United States, excessive alcohol consumption is the third main preventable cause of death and is associated with adverse health consequences such as liver cirrhosis and different types of cancer [4].

Alcohol and its metabolites cause generalized disturbances in various organ systems such as the nervous, digestive, urinary and male reproductive systems, especially in the prostate gland [5-7].

Animal models have contributed to the elucidation of many biological, biochemical, physiological and morphological aspects involved in the habit of consumption of alcoholic beverages by humans. Various experimental studies conducted on mice [8] and rats [9, 10] have emphasized the alcohol preference of some rodent lines which showed rapid metabolic adaptation to the initial effects of ethanol ingestion. This is the case of UChB rats which present acute and rapid tolerance to ethanol [11]. These rats, originated from Wistar rats, have gone through decades of selection by inbred crossings between animals characterized by low and high voluntary 10% ethanol consumption, called UChA and UChB rats, respectively [12].

In the male reproductive system, one of the consequences of alcoholism is hypogonadism which is observed both in man and in laboratory animals [13]. Ethanol is believed to act directly on the gonads by altering the synthesis of testicular testosterone [14-19], and also indirectly through changes in the hypothalamus-pituitary-gonadal axis [20]. In

the ventral prostate of rodents, both a direct and indirect effect of ethanol on the glandular epithelium has been suggested [21].

The harmful effect of alcohol on the male reproductive system has been demonstrated using various methods for the induction of alcoholism. The main morphological alterations were damage of testicular cells, reduction in the diameter of the seminiferous tubules, depression of serum testosterone levels, reduction in the weight of accessory sex glands, and significant decrease in the height of secretory epithelial cells of the seminal vesicles and ventral, lateral and dorsal prostate [22-31]. In the accessory sex glands, deleterious effects on organelles involved in secretory processes can be emphasized, such as dilatation of the cisternae of the granular endoplasmic reticulum [GER] and Golgi complex, rupture of the microvilli, and accumulation of lipid droplets in the cytoplasm of epithelial cells [21, 27-31].

The prostate is a male accessory sex gland that raises interest among researchers because it is frequently affected by different pathologies such as benign prostatic hyperplasia and cancer [32]. Epidemiological studies have demonstrated increase in the frequency of prostate cancer since 1998, which in many cases leads to the death of the patient [33]. Prostatic diseases, especially carcinoma, are known to be of an endocrine nature [34] and their frequency increases with age [35].

The development and maintenance of morphofunctional activity of the prostate are mainly regulated by testosterone, in addition to other hormones such as estrogen, whose mechanism of glandular action has not been completely established [36-38].

In rodents, the prostate is a complex gland formed by three pairs of lobes: ventral, lateral and dorsal lobes [39-43]. The ventral prostatic acini are lined with simple epithelium

with tall columnar cells situated on a distinct basement membrane [44]. The prostatic stroma, on the other hand, is a complex arrangement of stromal cells and extracellular matrix associated with growth factors, regulatory molecules and remodeling enzymes. Stromal cells and extracellular matrix characterize a microenvironment that regulates the growth and functional differentiation of adjacent cells, with each of these components playing an important role in the maintenance of tissue form and function [45-47]. In addition, epithelial-stromal interaction plays important role in maintaining the structure and function of the prostate gland [48]. Recent studies have demonstrated that epithelial-stromal interaction is the principal factor in the progression of prostate carcinoma [47, 49].

The consequences of alcohol toxicity on the prostate are complex and doubts remain regarding the morphophysiology of the stroma and its interaction with epithelial cells in response to chronic alcoholism. Thus, the aim of the present study was to analyze the morphophysiological alterations that occur in the secretory and stromal portion of the ventral lobe of the prostate of UChB rats in response to abusive alcohol use and alcohol abstinence, as well as to associate these results with prostatic pathogenesis.

MATERIAL AND METHODS

Animals and Procedures

Ten Wistar rats (control group) and 20 UChB rats (alcoholic and abstinent groups) aged 3 months were used. The control group received tap water as the liquid diet, the alcoholic group received ethanol diluted to 10º Gay Lussac (G.L., 10 ml ethanol/100 ml solution) for 150 days, and the abstinent group received 10º G.L. ethanol for 120 days and then tap water like the control group for more 30 days. All animals received Nuvilab® CR chow *ad libitum* as the solid diet. It should be noted that 1 g solid diet supplies 2.7 kcal of energy and 1 g ethanol contains 7.1 kcal. Liquid intake was measured daily. Solid intake and body weight were measured weekly. At the end of each treatment period, the animals of each group were weighed, anesthetized with Francotar®/Virbaxyl® (Virbac, Brazil) (1:1) and then submitted to cardiac puncture to obtain blood samples for hormone measurement. The testes, seminal vesicles, coagulation glands and ventral lobes of the prostate were collected and weighed on a Sartorius 2434 analytical scale. The samples of the ventral lobes of the prostate were then analyzed by histochemistry, immunohistochemistry and transmission and scanning electron microscopies.

Light Microscopy

The ventral prostate was collected, fixed by immersion in Bouin's solution and formaldehyde 10%, embedded in paraplast (Paraplast Plus, Brazil) and methacrylate resin

(Historesin Embedding Kit, Leica, USA), cut into 3-μm thick sections, and submitted to the following staining procedures: a) hematoxylin and eosin, b) Picrosirius red [50], and c) Gomori's silver impregnation for reticulin [51]. The thin sections were photographed with a Nikon Eclipse E-400 photomicroscope.

Immunohistochemistry

Prostate samples were embedded in tissue freezing medium (TBS, Durham, NC), frozen in liquid nitrogen, and cut into 7-μm thick sections with a Microm cryostat. The sections were collected, fixed with ice-cold methanol (3 min) and acetone (3 min), and air-dried for 30 min, followed by fixation in 4% paraformaldehyde for 10 min and rinsing with PBS. Next, the specimens were incubated with blocking solution (4% BSA and 1% Triton X-100, in PBS; Sigma, St. Louis, MO) for 1 h at room temperature to block nonspecific binding. Primary rabbit anti-type I and anti-type III collagen antibodies (Chemicon International, Inc., Temecula, CA) were diluted in blocking solution according to manufacturer instructions and applied to the sections overnight at 4ºC. The slides were washed three times for 5 min with PBS and the material was incubated with a secondary fluorescein-conjugated antibody (anti-rabbit IgG, FICT, Sigma Chemical Co.) diluted 1:100 in blocking solution. The sections were mounted in DABCO (Sigma Chemical Co.) solution and observed under a confocal microscope (BioRad MRC1024UV).

Stereological Procedures

Five rats from each group were used. Epithelial and stromal areas in the ventral lobe of the prostate were measured (25 fields per animal). The microscopic field was scanned at 200X magnification using a calibrated eyepiece (Filar Micrometer, Nikon). The areas were measured using the Image-Pro Express 4.0 computerized image analysis system. Cellular, cytoplasmic and nuclear volumes were measured in sections stained for light microscopy. Nuclear volume was recorded as the average of 100 measurements per animal. Long and short axes were measured and the mean volume was calculated considering nuclei as ellipsoids. The sections were analyzed with an ocular micrometer coupled to a Zeiss microscope equipped with a 100X objective. For the determination of cytoplasmic volume, an eyepiece with a 400 grid coupled to a 100X objective was used. Points on the cytoplasm and nuclei were counted in 10 areas per group and the cytoplasmic and nuclear fractions were obtained. These data and the nuclear volume were used to estimate the cytoplasmic volume for each animal [52]. Cell volume was calculated by summing the nuclear and cytoplasmic volumes.

Transmission Electron Microscopy

Five animals per group were perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer through the left ventricle [53]. The ventral lobe of the prostate was collected and fixed by immersion in the same fixative. The material was then dehydrated in a graded acetone series, the tissue was embedded in Araldite resin (Polysciences, USA), and 0.5-µm

thick sections were stained with Toluidine blue and prepared for light microscopy in order to choose specific areas for transmission electron microscopy analysis. Ultrathin sections were obtained with an LKB ultramicrotome and contrasted with uranyl acetate [54] and lead citrate [55]. Electron micrographs were obtained with a LEO 906 electron microscope.

Scanning Electron Microscopy

Specimens were prepared as described for transmission electron microscopy up to the last dehydration step in absolute acetone. The material was then submitted to critical point drying in liquid CO_2 using a CPD 030 Balzers apparatus. The specimens were coated with gold using an SCD 050 Balzers sputtering device and observed under a JEOL JSM-5880LV scanning microscope.

Hormone Measurement

Blood samples from five animals of each group were used for the determination of testosterone by RIA using the Coat-a-Count Total Testosterone Kit (Diagnostic Products Corporation, Los Angeles, CA, USA). Blood was collected at the end of the 150-day experiment.

Statistical Analysis

Data were analyzed statistically by analysis of variance and the Tukey multiple range test, with the level of significance set at 5% [56].

RESULTS

Nutritional Analysis and Body Weight

No difference in body weight gain was observed between the three experimental groups. On the other hand, liquid and solid intakes were lower in the alcoholic group and higher in the abstinent group compared to the control group (Table 1). The consumption of 10% ethanol differed between alcoholic and abstinent animals, with a mean intake of 2.76g ethanol/kg body weight/day in the alcoholic group and of 2.05g ethanol/kg body weight/day in the abstinent group.

The testes were significantly reduced in the alcoholic and abstinent groups compared to the control group. The weight of the seminal vesicles and coagulating glands was lower than in the control group, although this difference was not statistically significant (Table 1).

Light Microscopy, Immunohistochemistry and Stereology

Control Group

The prostatic ventral lobe was characterized by acini of different sizes and infolded mucosa (Figs. 1A, 1B and 1D). The secretory epithelium was simple with tall columnar cells and a basal nucleus (Figs. 1A and 1C). The prostatic stroma showed thin and short type I collagen fibers (Figs. 1B, 1C and 2A) often underlying the epithelium and intermingled with smooth muscle cells and fibroblasts. In addition, reticular fibers (type III

collagen) were observed underlying the epithelium around prostatic acini (Figs. 1D and 2D). The epithelial glandular area was approximately three times larger than the stromal area (Table 2).

Alcoholic Group

The prostatic acini were smaller and contained less infolded mucosa than observed for the control group (Figs. 1F and 1G / Table 2). A drastic reduction in the total volume of secretory epithelial cells was noted, with the nucleus occupying a large part of the cytoplasm (Fig. 1G and Table 2). Focal areas of secretory epithelial cell stratification were observed characterizing prostatic intraepithelial neoplasia [PIN] (Fig. 1M and 1N). The secretory epithelium showed evaginations consisting of cell masses projecting to the stroma, but the basement membrane was intact (Figs. 1H). Enlargement of collagen fibers [type I collagen] was noted in the prostatic stroma, indicating hypertrophy of stromal fibrillar elements not only in the underlying epithelium but also throughout the stromal area (Figs. 1F, 1G and 2B). In addition, an increase in smooth muscle cell layers was observed around the acini, especially in the case of structural disorganization (Fig. 1F). Cellular and fibrillar elements of the glandular stroma were increased, as demonstrated by only 1.5 times smaller stromal area than epithelial one (Table 2). Reticular fibers [type III collagen] were increased in thickness, showing undulated aspect (Figs. 1H and 2E). These fibers were found throughout the stromal area. In addition, focuses of inflammatory cells were frequently observed in the glandular stroma (Fig. 1E), mainly in areas without high epithelial reduction.

Abstinent Group

The cell volume was higher than that obtained for the alcoholic group, showing numerical recovery (Fig. 1I and Table 2). Nevertheless, enlargement of collagen fibers [type I collagen] was noted close to the epithelium and around all prostatic stromal areas as observed in the alcoholic group (Fig. 1J, 1K and 2C). Increase of the reticular fibers [type III collagen] was noted but was not as evident as in the alcoholic group (Figs 1L and 2F). Therefore, the distribution of reticular fibers close to epithelial cells was similar to that observed in the control group (Fig. 1L). Inflammatory cells were intermingled with fibrillar elements, especially collagen fibers (Fig. 1K). PIN and epithelial evagination were observed as in the alcoholic group, but these morphological characteristics were less frequent (Fig 1J). The glandular epithelial area was only 1.7 times larger than the stromal one.

Scanning and Transmission Electron Microscopy

Control Group

The prostatic epithelium consisted of tall columnar cells with a basal nucleus and a clearly visible nucleolus (Figs. 3A and 3B). The cellular cytoplasm showed GER with parallel and flattened cisternae (Figs. 3A and 3B) and a well-developed Golgi complex (Fig. 3E) in the perinuclear and supranuclear regions. Secretory vacuoles containing secretion granules were identified in the apical cytoplasm (Fig. 3C). Basal cells were noted between epithelial cells resting on a clearly visible and intact basal lamina (Fig. 3D). Thin

smooth muscle cells underlying the secretory epithelium were observed in the glandular stroma (Fig. 3D).

A close juxtaposition of cells was observed on the epithelial luminal surface (Fig. 3F). All epithelial cells presented uniformly distributed microvilli covering the cell surface (Fig. 3F). In addition, accumulation of glandular secretion could be noted (Fig. 3F).

Alcoholic Group

The secretory epithelium consisting of cuboidal cells and irregular basal nucleus was atrophic and showed a reduction of cell cytoplasmic volume (Fig. 4A). Proliferation of epithelial cells and intercellular spacing with PIN-like morphology were observed in some glandular regions (Fig. 4B). Dilatation of GER and Golgi complex cisternae were noted in the supranuclear region (Figs. 4C and 4E, respectively). Occasional points of discontinuity of the basal lamina were noted, in addition to an irregular distribution of subepithelial collagen fibers (Fig. 4D). In the apical region, some secretory vacuoles were observed (Fig. 4C).

The intercellular spacing was increased in these animals (Fig. 4F). However, the convex form of the apical cell and the distribution of microvilli on the luminal surface were similar to those of the control group (Fig. 4F).

Abstinent Group

Abstinent animals showed recovery of prostatic epithelial cell volume compared to alcoholic animals. The basal nucleus recovered its regular shape comparable to that of the

control group (Fig. 5A). In the supranuclear region, the GER cisternae were flattened but some of them were dilatated, characterizing intermediate morphological aspects between the control and alcoholic groups (Fig. 5B). Golgi complex were dilated (Fig. 5B). In addition, secretory vacuoles were identified in the cell apical cytoplasm (Fig. 5C). Nevertheless, the prostatic stroma continued to be hypertrophic (Fig. 5D).

As in the control group, the prostatic cells of abstinent animals were closely juxtaposed, reducing the intercellular space (Fig. 5E). The microvilli were uniformly distributed on the luminal cell surface as observed for the other groups (Fig. 5E).

Hormone Analysis

A significant reduction of serum total testosterone levels was observed not only in the alcoholic group but also in the abstinent group compared to control animals. This reduction was more prominent in the alcoholic group (Fig. 6).

DISCUSSION

In the present study, atrophy of male genital organs such as testes, seminal vesicles and coagulation glands was observed in the alcoholic and abstinent groups compared to control animals, with hypogonadism being significant in the alcohol-treated groups compared to control. In contrast, the weights of the seminal vesicles and coagulation glands of alcohol-treated animals, although lower, did not differ significantly from those obtained from control animals. Atrophy of the testes and accessory sex glands has been described in chronically alcohol-treated animals. According to Tadic et al. [19], Wistar rats showed marked testicular reduction after ethanol ingestion for 3 months. Furthermore, Martinez et al. [12], studying UChB rats voluntarily consuming 10% ethanol for 150 days, confirmed the occurrence of testicular atrophy. In that study, the authors found no difference in seminal vesicle weight between UChB rats and control animals. Cagnon et al. [31] also observed no marked differences in the weight of the seminal vesicles and coagulation glands between control mice (C57B1/6J) and mice treated with 6% ethanol for 120 days despite significant atrophy of the glandular secretory compartment in the later. Thus, it can be concluded that in alcoholic rats ethanol exerts toxic effects not only on the macroscopy of the gonads but also on that of the accessory sex glands.

The present results showed that all experimental animals gained body weight throughout the experimental period. Although those values were lower in the alcoholic and abstinent groups compared to the control group, this difference was not statistically significant. In addition, total calorie intake from the ration was numerically higher in the control and abstinent groups than in alcoholic animals, with mean daily intakes of 149.6

kcal/kg$^{0.75}$, 157.6 kcal/kg$^{0.75}$ and 138.4 kcal/kg$^{0.75}$, respectively. According to the National Research Council [57], the daily energy requirement for the maintenance of body functions of adult rats is approximately 110 kcal of digestible energy per kg$^{0.75}$ body weight. In the literature, several morphological studies conducted on alcoholic animals, especially involuntarily alcohol consuming ones, have raised concern regarding the nutritional status of these animals. These studies demonstrated that chronic alcoholic animals presented deficient weight gain, with weight gain being numerically lower than in controls [58-60]. Campana et al. [61] reported that in the state of protein malnutrition the animals presented disturbances such as hair loss or altered hair distribution, diarrhea and edema, in addition to marked weight loss. Furthermore, Mardones and Quintanilla [62] demonstrated a natural tendency of UChB and UChA rats to ingest alcohol based on the administration of bromocriptine, which acts on ethanol satiety in rats. In that experiment, a specific reduction in alcohol consumption was observed during treatment with the drug, without alterations in ration intake. Ethanol consumption was resumed after the discontinuation of bromocriptine treatment. Thus, it was concluded that the amounts of energy ingested by the animals studied were adequate for the development and maintenance of body requirements. Also, they showed voluntary ethanol consumption, a fact that qualified them as an excellent model for the study of chronic alcoholism.

Chronic alcoholic animals showed marked structural and ultrastructural alterations in both secretory epithelium and glandular stroma. These changes included epithelial atrophy, PIN, epithelial evaginations projecting to the stroma, alterations in the biomembrane system of organelles involved in the glandular secretory process, an increase and irregular distribution of subepithelial collagen fibers (types I and III), altered smooth

muscle morphology and presence of inflammatory cells in the prostatic stroma. Experimental studies employing different alcohol doses and treatment times have demonstrated the damaging effects of abusive ethanol use on the accessory sex glands, including epithelial atrophy and alterations in the cell organelles involved in the secretory process [12, 29, 31]. On the other hand, no prostatic focal intraepithelial proliferation was observed in these studies. PIN has been clinically indicated as a precursor lesion of invasive adenocarcinoma [63-66]. The high clinical incidence of prostatic cancer identified in positive biopsies obtained from patients with PIN, especially high grade PIN, confirms the tendency of PIN to progress to malignancy [63-65]. Thus, it can be concluded that ethanol was a harmful drug not only to the glandular secretory process but also caused prostatic pathogenesis which might be associated with later processes of glandular malignancy.

In the specialized literature, the stromal microenvironment is known to be dynamic and to directly influence the differentiation of prostatic epithelial cells and glandular growth and function, in addition to actively participating in tissue repair in response to injuries [47, 67]. Furthermore, epithelial-stromal interaction plays an important role in the maintenance of the structure and functioning of the prostate gland [48]. It is known that an imbalance in the interaction of the glandular compartments favors the onset and development of prostate carcinoma [37]. According to several investigators, stromal cells together with tumor cells respond to androgens and growth factors, leading to the interruption of epithelial-stromal homeostasis, an event that definitely triggers processes of cell growth, angiogenesis, apoptosis, and tumor metastases [68, 69, 67].

Vilamaior et al. [51], investigating the prostate gland of rats after castration, reported that smooth muscle cells showed an altered phenotype in response to reduced

serum testosterone levels, with this alteration not only involving the synthesis and secretion of extracellular matrix components but also the active remodeling of fibrillar components of the stroma. An altered smooth muscle cell phenotype was also observed by other investigators both in remodeling tissues in response to different injuries and in prostate carcinomas. Such alteration characterizes these cells as myofibroblasts, which are secretory cells of an intermediate phenotype between fibroblasts and smooth muscle cells [47, 70, 37]. So, it can be concluded that in the present study, abusive ethanol consumption caused a change in smooth muscle cells which assumed a secretory phenotype and were responsible for the hypertrophy of stromal fibrillar elements.

The presence of inflammatory cells in the prostate stroma of alcoholic animals has not been emphasized in the literature. However, the prostate chronic inflammatory processes are associated with both postatrophic hyperplasia and simple focal atrophy [71]. According to Billis [72], chronic prostatitis is generally caused by bacteria. However, among the different types of nonspecific chronic prostatitis the noninfectious forms are the most frequent and are a constant finding in benign prostatic hyperplasia. This author also reported that this process of inflammation is the result of extravasation of prostatic secretion into the stroma after obstruction of the ducts. Current studies have demonstrated a relationship between inflammatory infiltrates and tumor maintenance and progression [73, 74]. According to Lin & Pollar [74], inflammatory cells, especially leukocytes, when present in the tumor microenvironment, promote the production of diverse growth factors, proteases and angiogenic mediators, thus permitting tumor maintenance and progression. Thus, the presence of inflammatory cells concomitantly with the occurrence of PIN and stromal hypertrophy observed in the present study suggests an active participation of these

cells in the destructuring of the epithelial-stromal interaction. On the other hand, the occurrence of inflammatory cells might have been secondary to the abusive use of alcohol, originating from the process of epithelial atrophy and stromal hypertrophy which probably caused extravasation of prostatic secretion into the stroma, indicating the possible presence of an inflammatory process.

Abstinent animals showed quantitative volume recovery of the secretory epithelium. However, similar to the alcoholic group, the prostatic stroma was found to be hypertrophic and inflammatory cells were present. On the other hand, PIN and epithelial evaginations were observed only occasionally. Cagnon et al. [29], studying Wistar rats submitted to experimental chronic alcoholism for periods ranging from 60 to 300 days, observed recovery of epithelial height in the alcoholic group after interruption of ethanol ingestion for 60 days, similar to the present findings. However, the same authors noted that the alterations in the organelles involved in the glandular secretory process persisted. With respect to stromal alterations, studies correlating the effects of alcoholism with changes in the prostatic stroma are scarce. However, the fact that no complete recovery of prostatic tissue was observed in abstinent animals indicates continuous glandular destructuring.

Hormone measurement revealed a significant decrease in serum testosterone levels in both alcoholic and abstinent rats compared to control animals. Different investigators have shown a reduction of serum testosterone levels in animals submitted to the ingestion of various alcohol doses, with these alterations being attributed to a direct action of ethanol on tissues as well as to an indirect action through imbalance of the hypothalamus-pituitary-gonadal axis [75, 19]. Martinez et al. [12] reported a significant reduction of serum testosterone in UChB rats voluntarily consuming 10% ethanol compared to control UChA

and Wistar rats. Tadic et al. [19] also observed a significant decline in serum testosterone in Wistar rats submitted to chronic alcoholism for periods ranging from 30 to 90 days. These authors characterized a direct relationship between the testicular atrophy of these animals and plasma testosterone levels. Although basically regulated by androgens, it is now known that the prostate also responds to estrogen hormones [37]. The relationship between androgens and estrogens has been considered to be fundamental for the maintenance of prostatic glandular equilibrium [67]. Furthermore, different investigators have demonstrated that androgen depletion and the consequent change in the estrogen/testosterone ratio result in intensive tissue remodeling, especially of the prostatic stromal component. This imbalance has been directly associated with the development of benign prostatic hyperplasia and prostate cancer [76, 37, 38]. Although estrogen levels were not measured in the present study, we believe that alterations in the estrogen/testosterone ratio resulting from the reduction of serum testosterone levels certainly contributed to the processes of epithelial atrophy and stromal hypertrophy observed in these animals.

Recent epidemiological studies have been conducted to correlate the effects of alcoholism with the possibility or an increased risk of developing prostate cancer [77], but no direct relationship was observed. However, the present results indicate that ethanol causes intensive stromal disorganization characterizing the interruption of epithelial-stromal homeostasis, a fact that certainly qualifies this drug as an inductor of epithelial proliferation which predisposes the organ to processes of glandular malignancy. Thus, chronic alcoholism can be considered as effective trigger of prostatic pathologies, including prostate carcinoma.

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FIGURE LEGENDS

Figure 1 – Photomicrographs of the ventral prostate of control (A-D), alcoholic (E-H, M, N) and abstinent rats (I-L). **A** – Folded prostatic acinus surrounded by a thin layer of smooth muscle cells (arrowhead) HE: x215. **B** – Prostatic stroma containing thin and scarce collagen fibers (arrow) and smooth muscle cells (empty arrowhead). Picrosirius-hematoxylin: x215. **C** – Simple secretory epithelium (Ep) with tall columnar cells. Thin collagen fibers adjacent to the epithelium (arrow). Picrosirius-hematoxylin: x430. **D** – Reticular fibers (wide arrow) accompanying the smooth muscle bundles that surround the prostatic acini. Gomori reticulin: x430. **E** – Acini showing reduced lumen (L) and areas of epithelial cell proliferation (Ep). Glandular stroma with inflammatory cells (star). HE: x215. **F** – Acini showing poorly folded mucosa. Accumulation of collagen fibers in the prostatic stroma (arrow). Note the increase in the smooth muscle cell layer around the acini that showed cell involution (empty arrowhead). Picrosirius-hematoxylin: x215. **G** – Epithelial atrophy (Ep). Apparent increase in the amount of collagen fibers (arrow). Presence of inflammatory cells (star). Picrosirius-hematoxylin: x430. **H** – Epithelial evagination (star) and stromal accumulation of reticular fibers with an undulated aspect. Glandular lumen (L). Gomori reticulin: x430. **I** – Simple columnar secretory epithelium surrounded by a thick layer of smooth muscle cells (empty arrowhead). HE: x215. **J** – Epithelial evagination (star). Prostatic stroma (St). Glandular lumen (L). Picrosirius-hematoxylin: x215. **K** – Columnar epithelial cells showing recovery of cell volume (Ep). Stroma with accumulation of collagen fibers (short arrow). Inflammatory cells among collagen fibers (long arrow). Picrosirius-hematoxylin: x430. **L** – Folded secretory epithelium. Note the presence of thick reticular fibers (wide arrow). Gomori reticulin: x430.

M and **N** – Prostatic acini showing intraepithelial neoplasia (arrows). Prostatic stroma (St). Glandular lumen (L). Picrosirius-hematoxylin: x215 and x430, respectively.

Figure 2 – Prostatic ventral lobe. Immunohistochemistry for type I collagen fibers (A-C). **A –** Control group. Thin sheet of collagen I fibers (arrow) underlying the secretory epithelium and around blood vessels. **B –** Alcoholic group. Accumulation of collagen I (arrow) underlying the secretory epithelium and around micro-acini. **C –** Abstinent group. Collagen I fibers (wide arrow) underlying the secretory epithelium. Stromal accumulation of collagen I fibers (arrow). Prostatic ventral lobe. Immunohistochemistry for collagen type III fibers (D-F). **D –** Control group. Stroma containing small agglomerates of type III fibers (arrows). **E –** Alcoholic group. Collagen III distributed both at the base (arrow) of the epithelium and at other points (wide arrows) of the prostatic stroma. **F –** Abstinent group. Collagen type III fibers (arrows) throughout the prostatic stroma. Note the epithelial evagination. Bar = 50 μm.

Figure 3 – Electromicrographs of the ventral prostate of control rats. **A** and **B** – General view of the secretory epithelium. Simple epithelium with tall columnar cells intermingled with basal cells (Bc). Oval nucleus (N) with regular contours. Note the granular endoplasmic reticulum (GER) cisternae in the perinuclear cytoplasm containing material of low electron density. Lumen (L). Bar = 1.0 μm. **C** – Detail of the apical region. Short and scattered microvilli (MV) cover the cell surface. Secretory vacuoles containing granules of different electron densities are present. **D** – Detail of the basal region showing a basal cell (Bc) resting on a clearly visible basal lamina (↖). Stroma (St) containing smooth muscle

cells (SM). Bar = 1.0 μm. **E** – In the supranuclear region, note the Golgi complexes with flattened and thin vesicles arranged in a parallel fashion (↖). Bar = 1.0 μm. **F** – Detail of the luminal surface of the secretory epithelium. Note the integrity of the secretory epithelium with reduced intercellular space between cells (arrow) and the regular distribution of microvilli on the convex apical surface of these cells. Secretion granules (asterisk). Bar = 10μm.

Figure 4 – Electromicrographs of the ventral prostate of alcoholic rats. **A** – Atrophied epithelium with involuted cells. Basal nuclei (N) with irregular contours. Intercellular spacing (✱). Lumen (L). Bar = 1.0 μm. **B** – Occurrence of prostatic intraepithelial neoplasia (PIN). Irregularly shaped nuclei (N). Intercellular spacing (✱). Prostatic stroma (St). Bar = 1.0 μm. **C** – Details of the supranuclear and apical regions. Dilatation of granular endoplasmic reticulum (GER) cisternae. Eventual secretory vacuoles of different electron densities (V) in the apical region of the cell. Small and scattered microvilli (MV) cover the cell surface. Glandular lumen (L). Bar = 1.0 μm. **D** – Detail of the basal region. Apparent discontinuity of the basal lamina. Stroma (St) containing scattered collagen bundles (Col). Irregular smooth muscle cell (SM) showing a spiny aspect. Epithelial cell nucleus (N). Bar = 1.0 μm. **E** – Detail of supranuclear region. Dilatation of Golgi complex (↖). Bar = 1.0 μm. **F** – Clearly visible intercellular spacing (arrow). Simple epithelium with a spherical nucleus (N). Clearly visible layer of smooth muscle cells (SM). Bar = 10 μm.

Figure 5 – Electromicrographs of the ventral prostate of abstinent rats. **A** – General view of the secretory epithelium with columnar cells. Basal nucleus (N) with regular contours. Secretory vacuoles (V) in the apical region of the cell. Small microvilli (Mv) cover the cell surface. Smooth muscle cells (SM). Lumen (L). Bar = 1.0 μm. **B** – Detail of the supranuclear region. Note the granular endoplasmic reticulum (GER) with flattened cisternae. Some of them were dilated (▲). Dilatation of the Golgi complex (↖). Cell nucleus (N). Bar = 1.0 μm. **C** – Apical region. Dilatation of GER. Secretory vacuoles (V). **D** – Detail of the stromal region. Visible basal lamina (↖). Voluminous stroma (St) with a large number of cells. Smooth muscle cells (SM) showing projections with small vacuoles (v). Bar = 1.0 μm. **E** – Regular epithelium with juxtaposed cells (arrow). Convex apical surface consisting of cells covered in a continuous fashion with microvilli. Note the simple epithelium, nuclei (N) with irregular contours and a thick layer of smooth muscle cells (SM) in the adjacent stroma. Collagen bundles in the prostatic stroma (asterisk). Bar = 10 μm.

TABLES

Table 1. Mean ± standard deviation of body and organ weights [testis, seminal vesicle and coagulation gland] and water and chow intake.

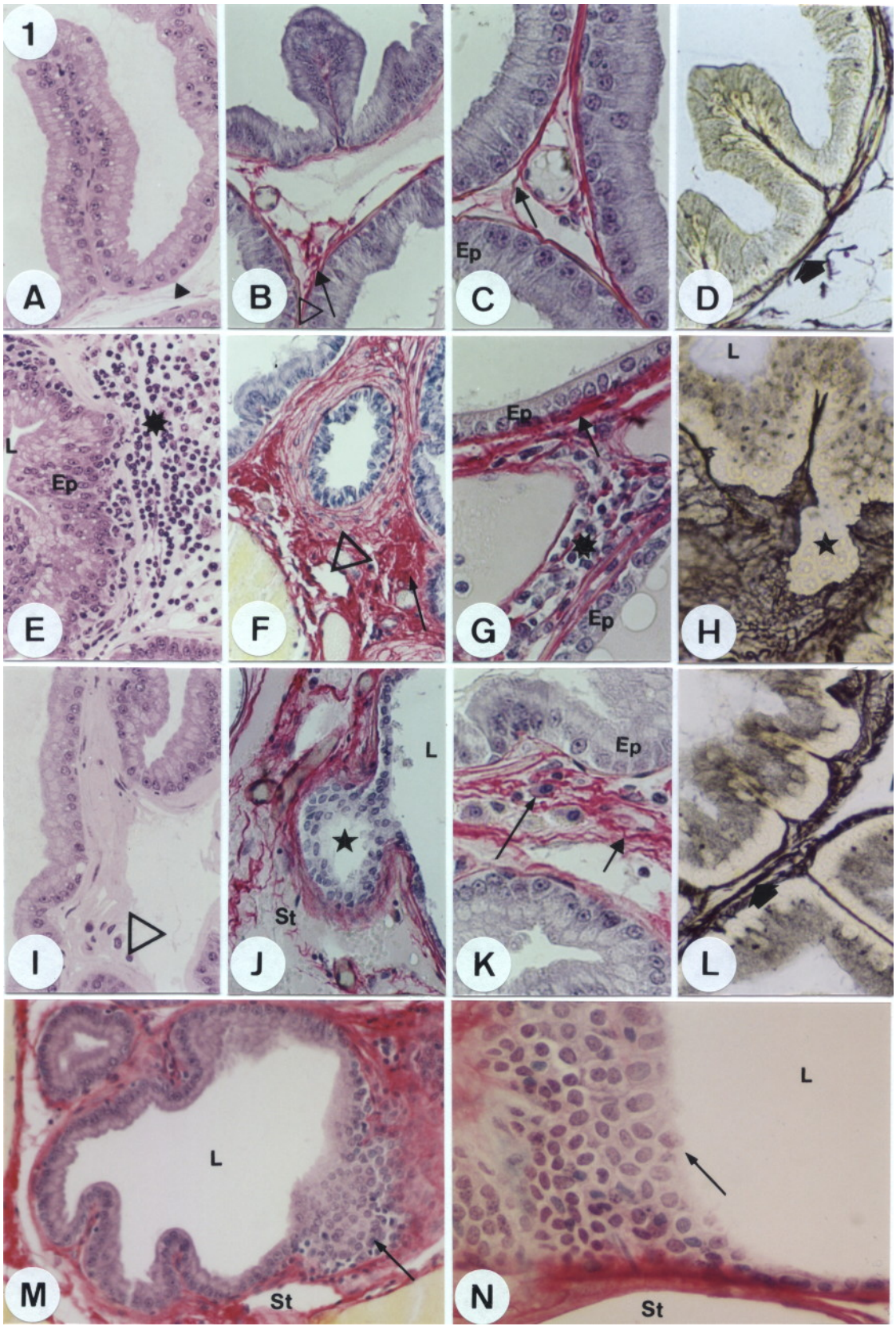
	Groups		
Variable	**Control**	**Alcoholic**	**Abstinent**
Body weight (g)	149.0 ± 28.4**a**	126.3 ± 24.0**a**	142.3 ± 30.6**a**
Chow intake (g/day)	27.4 ± 1.0**ab**	25.6 ± 1.9**a**	29.2 ± 2.3**b**
Water intake (ml/day)	44.8 ± 4.9**ab**	40.6 ± 3.7**a**	48.0 ± 3.6**b**
Testis (g)	2.05 ± 0.17**b**	1.59 ± 0.15**a**	1.67 ± 0.20**a**
Seminal vesicle (g)	1.08 ± 0.17**a**	1.07 ± 0.15**a**	0.90 ± 0.22**a**
Coagulation gland (g)	0.12 ± 0.02**a**	0.11 ± 0.02**a**	0.11 ± 0.02**a**

a, b: Means followed by the same letter did not differ significantly (Tukey multiple range test, $P < 0.05$).

Table 2. Mean ± standard deviation of epithelial and stromal areas and nuclear, cytoplasmic and cell volumes.

	GROUPS		
Variable	**Control**	**Alcoholic**	**Abstinent**
Epithelial area (μm^2)	$269.10^3 \pm 24.10^3$**b**	$226.10^3 \pm 31.10^3$**a**	$234.10^3 \pm 20.10^3$**ab**
Stromal area (μm^2)	$99.10^3 \pm 24.10^3$**b**	$142.10^3 \pm 31.10^3$**a**	$134.10^3 \pm 20.10^3$**ab**
Nuclear volume (μm^3)	100.8 ± 7.7**a**	90.0 ± 7.7**a**	96.8 ± 3.2**a**
Cytoplasmic volume (μm^3)	552.4 ± 87.1**b**	358.1 ± 76.1**a**	452.0 ± 55.7**ab**
Cell volume (μm^3)	653.3 ± 92.4**b**	448.1 ± 78.0**a**	548.9 ± 54.8**ab**

a, b: Means followed by the same letter did not differ significantly (Tukey multiple range test, $P < 0.05$).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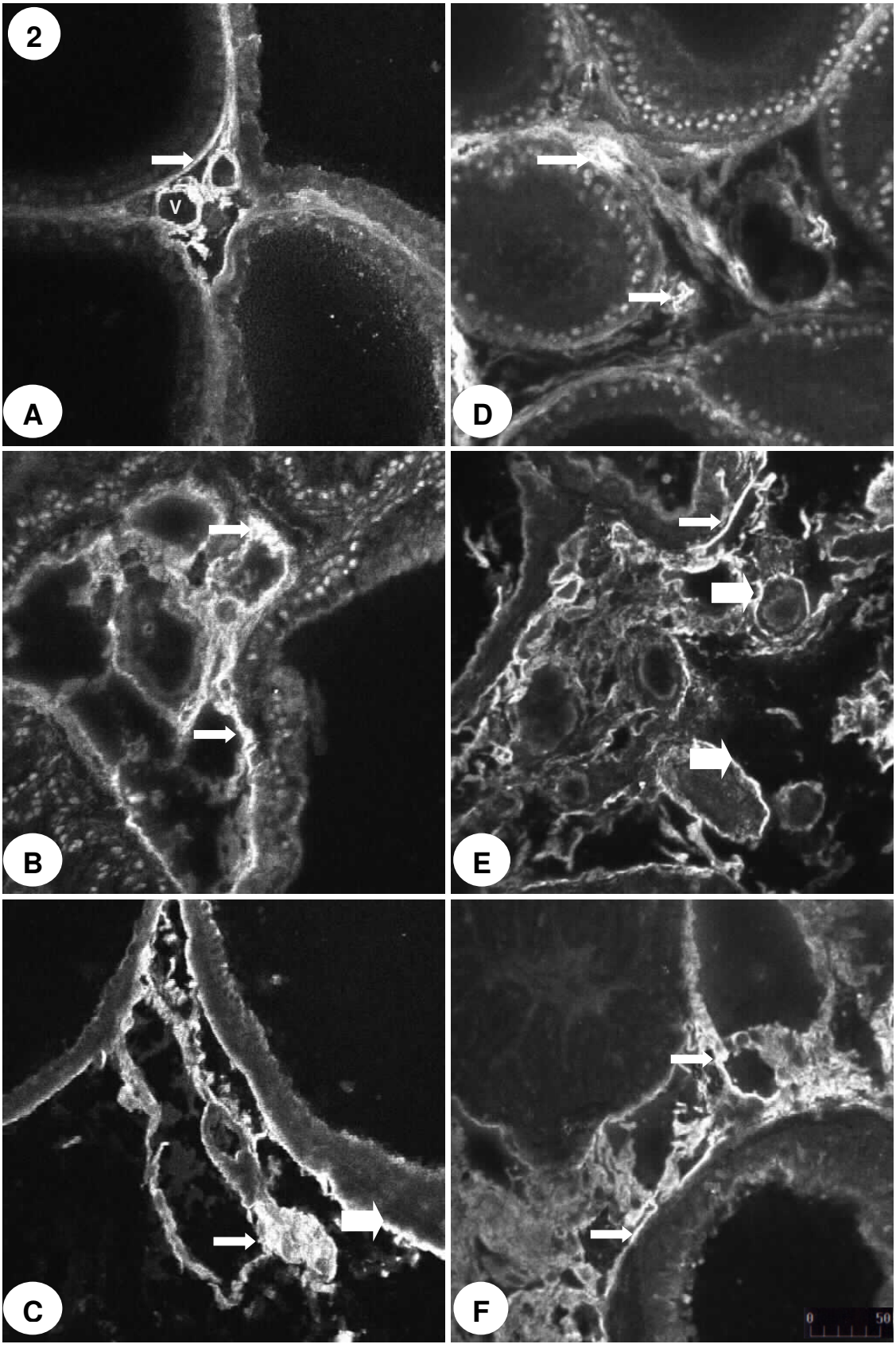
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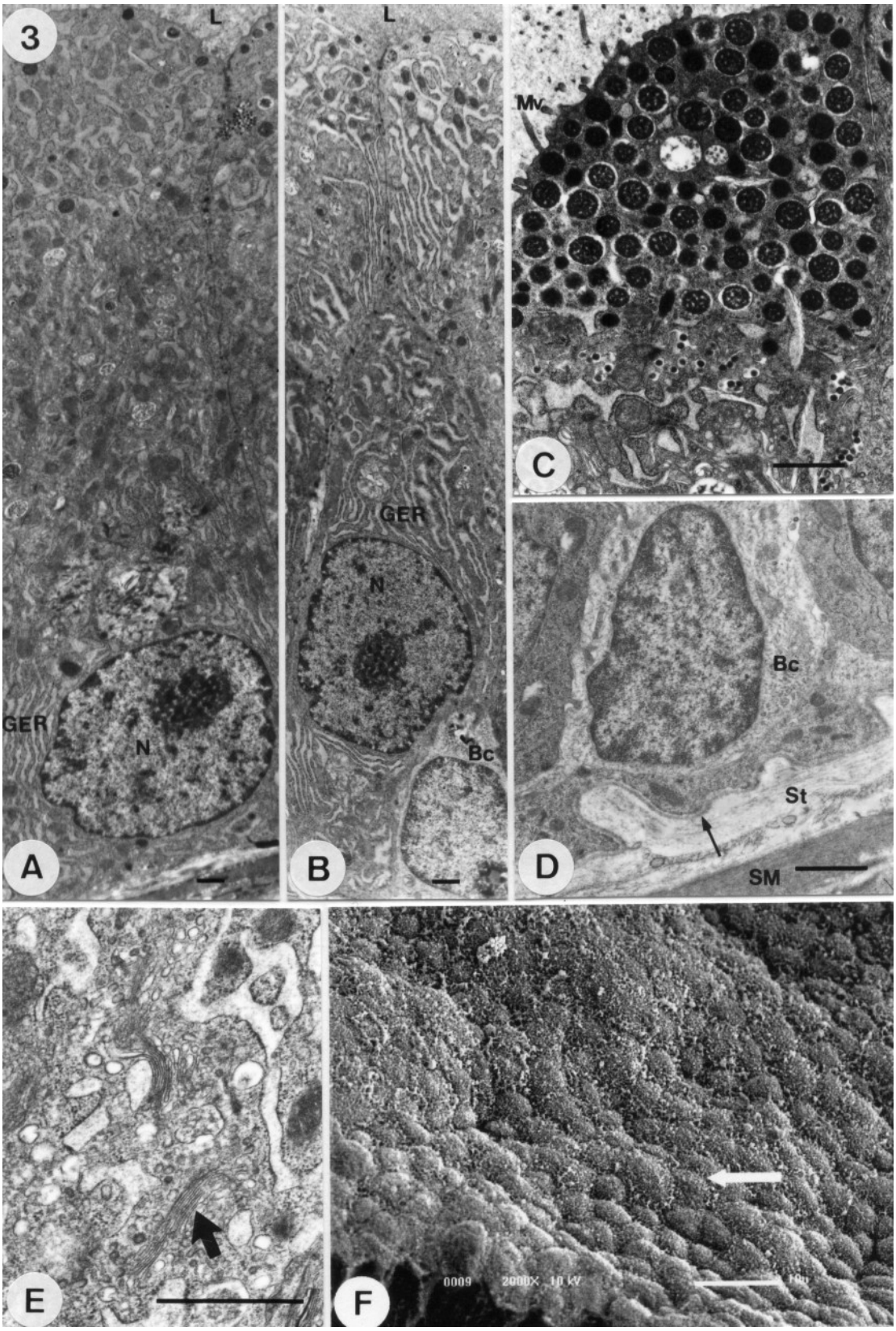
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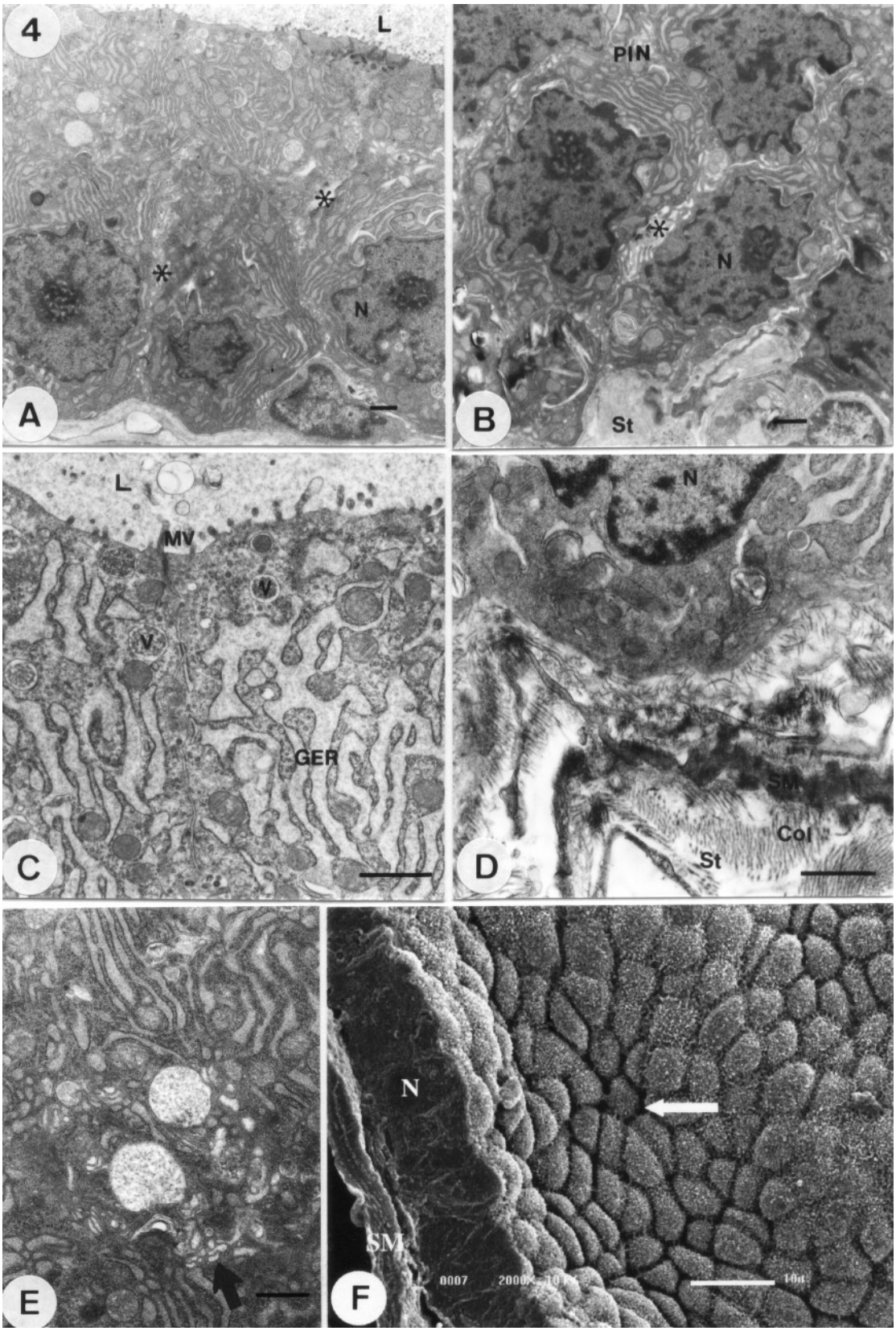
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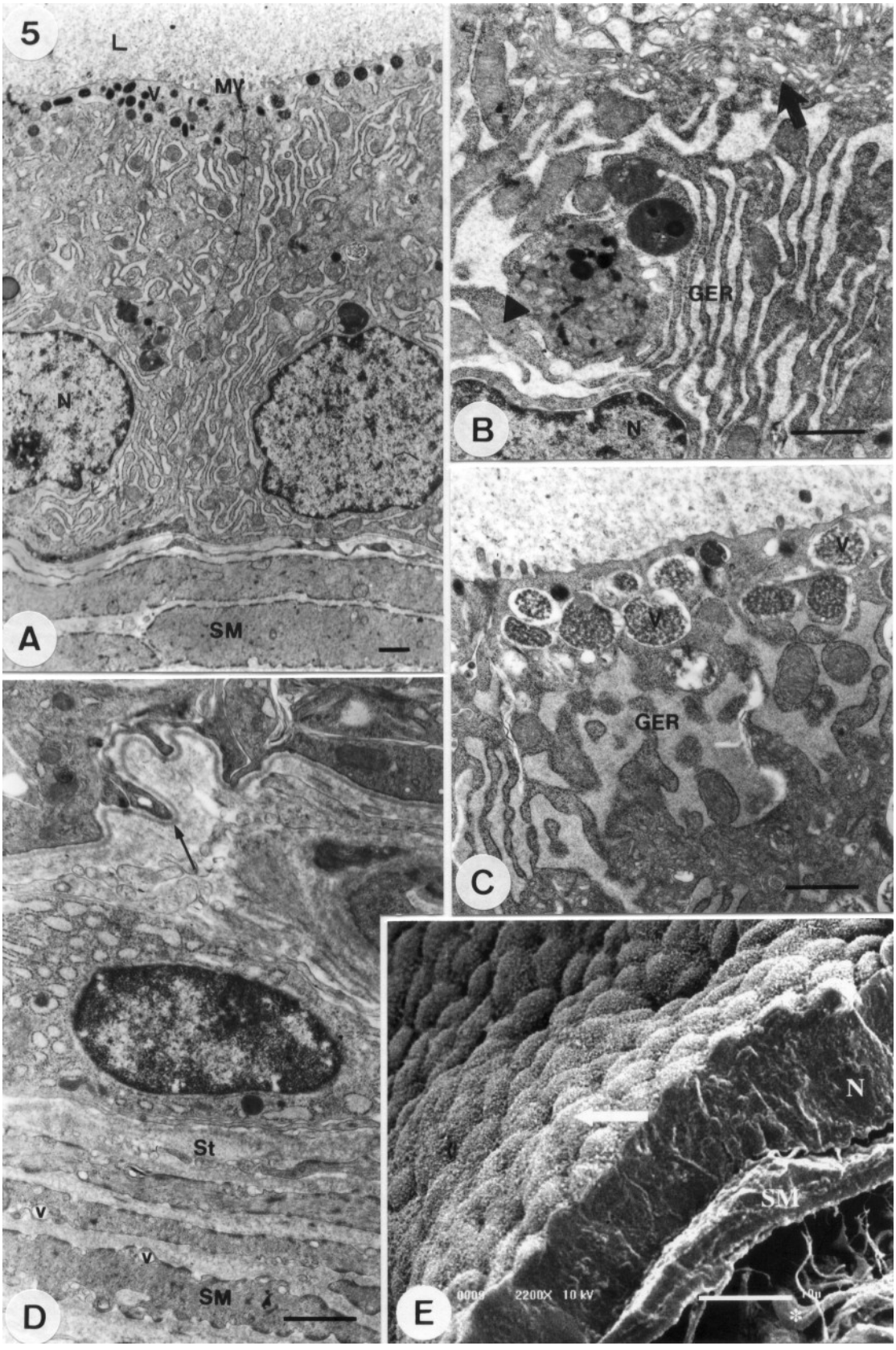
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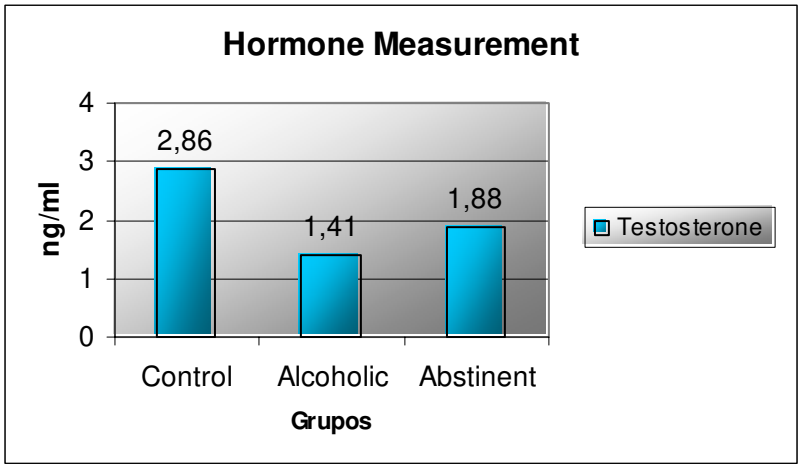
Figure 6 – Effects of 10% ethanol on serum total testosterone levels in control, alcoholic and abstinent animals after 150 days of treatment.



CONCLUSÕES GERAIS

1 – Os animais utilizados no presente estudo ingeriram quantidades adequadas de energia para o desenvolvimento e manutenção de suas necessidades corpóreas, mostrando que as alterações ocorridas foram provenientes do consumo etílico;

2 – O consumo crônico de etanol não só causa prejuízo ao processo secretor glandular, como também leva a eventos iniciais de lesões glandulares prostáticas;

3 – O consumo abusivo de etanol promove a ocorrência de infiltrados inflamatórios no estroma prostático;

4 – A interrupção do consumo etílico aos 120 dias não permite a completa recuperação tecidual prostática nos animais abstinentes, indicando a continuidade do processo de desestruturação glandular;

5 – A alteração das taxas estrógeno/testosterona provenientes da redução dos níveis de testosterona sérica, contribuem na ocorrência dos processos de atrofia epitelial e hipertrofia estromal dos animais tratados com etanol;

6 – O etanol leva a intensa desorganização estromal, caracterizando interrupção da homeostase epitélio-estromal, qualificando-o como fator indutor da proliferação epitelial, predispondo o órgão a processos de malignescência glandular. Assim, sugere-se que o alcoolismo crônico é um efetivo agente desencadeador de doenças prostáticas, incluindo o carcinoma prostático.

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